Subject: Registrant response addressing EPA-OPP-BPPD product characterization and toxicology issues communicated to registrant 09/29/2015 concerning 88877-EUP-E application.
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October 6, 2015
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(1) EPA-OPP-BPPD product characterization and toxicology issues: Address the research results reported in Dodson et al. (2014), Glaser & Meola (2010), Hughes et al. (2014) and Hussain et al. (2013). Specifically, Dodson et al. (2014) reported wAlbB infected *Culex tarsalis* may increase West Nile Virus infection rates and reduce gene expression of the REL1 (antiviral Toll pathway). While it is clear more research is necessary, please address this finding and how it relates to *Aedes aegypti* wAlbB releases and the potential or probability of increased infection rates and reduced immune gene expression of West Nile Virus in wAlbB infected mosquitoes. Also, please discuss your opinion on if these findings are due to host type, *Wolbachia* strain type, or both. Include a discussion on how other environmental factors such as temperature, as described by Hughes et al. (2014), may influence West Nile Virus infection rates, immune gene expression of antiviral pathways, and transmission potential. Reconcile the differences in the literature between Dodson et al. (2014), Hughes et al. (2014), Glaser & Meola (2010), and Hussain et al. (2013), which reported increased host resistance to West Nile Virus with *Wolbachia* presence in *Aedes aegypti* and *Culex quinquefasciatus*. The differences are most likely due to difference in strain and host uses. Unfortunately, no report was found by the EPA that specifically discusses *Aedes aegypti* wAlbB and West Nile Virus.

(1) Registrant response: The Dodson *et al.* (2014) study does not directly relate to wAlbB infection in *Ae. aegypti*. Differences include:

- The Dodson study pertains to a different mosquito genus (Culex). Our application is for *Ae. aegypti*.
- Culex mosquitoes are considered primary vectors of West Nile Virus (WNV). In contrast, *Ae. aegypti* are unlikely to acquire WNV, because it is unusual for *Ae. aegypti* to feed on host reservoir species, i.e., birds.
- The Dodson study is based on transient, somatic infections of Wolbachia, i.e., Wolbachia injected into the thorax of adults. This is unlike true Wolbachia infections that are transmitted maternally to eggs from the female. Transient somatic infections have not been shown to be predictive of true Wolbachia infections. As emphasized by Dodson *et al.* at the start of the discussion, "It should be noted that these experiments were performed with mosquitoes transiently infected in the somatic tissues with Wolbachia, rather than a stable maternally inherited infection. It remains to be seen whether a stable wAlbB infection will enhance WNV in a similar way."
- We will be releasing male mosquitoes only. Male mosquitoes do not bite and cannot vector WNV or other pathogens. Wolbachia is not transferred in the sperm to females during insemination. Thus, Wolbachia will not infect the targeted *Ae. aegypti* population through insemination with releases of incompatible male *Ae. aegypti* mosquitoes containing the

wAlbB Wolbachia infection. We will monitor for unintended establishment of Wolbachia at the release site.

The Glaser *et al.* (2010) study does not directly relate to wAlbB infection in *Ae. aegypti*. Similar to the Dodson *et al.* (2014) publication, it examines Wolbachia infection in Culex and the effect on WNV. The report contrasts with Dodson *et al.* (2014) in that Wolbachia infection is associated with reduced WNV transmission. Possible reasons include: "Enhancement may be dependent on the host-Wolbachia strain-pathogen system of interest" [Dodson *et al.* (2014)]. Notably, the two studies examine different species of Culex. The two studies also differ in that the Dodson *et al.* examine a transient somatic infection of Wolbachia and Glaser examines a true, maternally inherited Wolbachia infection.

The Hughes *et al.* (2014) discussion of temperature effects does not directly relate to wAlbB infection in *Ae. aegypti*. Specifically, Hughes *et al.* review temperature effects of a mouse malaria parasite in Wolbachia infected *Anopheles stephensi*. This is a different genus of mosquito and a different parasite species. *Ae. aegypti* is not a malaria vector. Also, as mentioned above, we intend to release male mosquitoes only. Without establishing Wolbachia in the targeted *Ae. aegypti* population, there is no potential or probability for a Wolbachia effect on malaria transmission in *Ae. aegypti*.

The Hussain *et al.* (2013) study does relate to Wolbachia infection in *Ae. aegypti*. While Hussain *et al.* examine different Wolbachia types, *i.e.*, wMel and wMelPop, the infections do not increase WNV infection in *Ae. aegypti*, and the wMelPop infection inhibits WNV in *Ae. aegypti*. Regardless, however, *Ae. aegypti* is not considered an important vector of WNV.

In conclusion, the report by Dodson et al (2014) does not directly relate to *Ae. aegypti* wAlbB releases. Because the targeted population does not become infected with Wolbachia, there is no potential or probability for reduced immune gene expression of West Nile Virus caused by Wolbachia. Furthermore, *Ae. aegypti* are not considered WNV vectors, because they do not feed on birds, which are the principal reservoir host.

(2) EPA-OPP-BPPD product characterization and toxicology issues: Provide statistical analysis of your data for the inferential power of your claim that 1 female wAlbB strain is expected per 250,000 individuals released. According to Calvitti et al. (2015), the current sexing technology is such that 1% female contamination is expected during male releases. Discuss how and why your sexing technology is superior to this.

(2) Registrant response: Our 'accidental female release rate' estimate is based on a protocol in which pools of male mosquitoes (*i.e.*, pools from which females were removed) were re-examined for females. Based on the observed error rate, we estimated one female per 250K males.

With the recent completion of the 2015 field season for EPA EUP No. 89668-EUP-1, our methods have been validated. Specifically, 90,000 male mosquitoes were delivered to NY, 163,000 mosquitoes were delivered to CA, and 500,000

mosquitoes were released in KY. Examination of male pools by collaborators in NY and CA allowed for an additional, external check for unintended females in males intended for release. These include experiments in which entire release pools were placed into cages for examination.

No females were identified in male release pools in KY, NY or CA, which is consistent with our expectations.

The Calvitti *et al.* (2015) publication does not appear to provide estimates of the current sexing technology. Instead it cites a Balestrino *et al.* (2014) publication, which in turn, cites a maximum rate of female release. We do not know what methods are used by Dr. Calvitti for removal of females or his accuracy rates.

(3) EPA-OPP-BPPD product characterization and toxicology issues: Address the report by Calvitti et al. (2015) that fertile crosses between wAlbA low density males and ARwP females demonstrate that mosquitoes with differing *Wolbachia* strains may still be fertile, and how this finding impacts *Wolbachia* male release strategies. Discuss how the finding in this study that the risk of bidirectional CI failure should be evaluated by sampling wild type males prior to field releases. And, if pertinent, present data on sampling of male pre-releases.

(3) Registrant response: The Calvitti *et al.* (2015) study does not directly relate to wAlbB infection in *Ae. aegypti*. Specifically, the report is about a different Wolbachia type (wPip) in a different mosquito species (*Aedes albopictus*).

Crosses of old wAlbB-infected *Ae. aegypti* males with wild type females remain completely incompatible and no egg hatch is being observed.

Even if there were full- or partial-loss of CI in old, wAlbB-infected males, little if any impact on our approach would be expected. Specifically, while two-week old males can be obtained in a laboratory setting, old males are generally thought to be irrelevant in the field. Very few males are expected to live two weeks in the field, and because new, young males are repeatedly released, any surviving old males would be overwhelmed by younger, more recently released males.